A STUDY OF GAS-PRODUCTION BY DIFFERENT STRAINS OF BACILLUS ABORTIVO-EQUINUS *

EDWIN S. GOOD AND LAMERT S. CORBETT

From the Department of Animal Husbandry, Kentucky Agricultural Experiment Station, Lexington

In our work on the etiology of infectious abortion in mares,[1] we found that the organism causing this disease, Bacillus abortivo-equinus, varied in its physiologic property of splitting some of the sugars, especially lactose and saccharose.

De Jong[2] makes the positive statement that the bacillus, which he isolated from the uterine exudate of aborting mares, does not ferment lactose, but that it does ferment saccharose. Meyer and Boerner[3] also state that the organism causing infectious abortion in mares does not ferment lactose and saccharose. Later, Van Heelsbergen,[4] working with De Jong, notes that the organism does not ferment lactose and saccharose.

Therefore, in order that more definite conclusions might be arrived at, considerable work needed to be done on this variable fermenting propensity, and the problem was taken up by us in the hope that some positive statement could be made concerning the gas-production of this organism.

As has been stated,[5] our laboratory placed this organism in Subgroup II of the colon-typhoid group, to which Bacillus enteritidis and the paracolon bacilli belong. As these organisms are all classified in the same subgroup, the reason for employing them in a comparative study of this nature can be readily understood.

It is interesting to note what some of our recognized authorities write concerning the splitting of lactose by bacilli classified in this subgroup. Take, for example, B. enteritidis (Gaertner). According to Buchanan,[6] "lactose is not fermented by the typical strains, although some strains have been reported by a few investigators to ferment this

* Received for publication November 24, 1915.

[1] Good and Corbett: Jour. Infect. Dis., 1913, 13, p. 53.
[2] Centralbl. f. Bakteriol., I, O., 1913, 67, p. 148.
[3] Jour. Med. Research, 1913-14, 29, p. 334.
[4] Inaugural Dissertation, 1913.
[5] Good: Bull. Ky. Agr. Exper. Sta., No. 165, 1912, p. 280.
[6] Veterinary Bacteriology, 1911, p. 269.

sugar, and the statement is commonly current in texts." Chester,[7] on the other hand, makes the unqualified statement that B. enteritidis forms gas in lactose broth. Herzog[8] also asserts that it ferments lactose, while Jordan[9] states that no gas or acid is formed from lactose.

J. Henderson Smith[10] has shown that organisms without the power to ferment certain carbohydrates may be so changed, after being grown for a number of generations on, or in, media containing these sugars, that they become true fermenters. He says: "In the year 1906, M. Neisser recorded the occurrence of a coli-form organism, which, while primarily a non-fermenter of lactose, could give rise to a fermenting strain when grown on lactose-agar. This organism was very carefully and thoroughly studied by Massini, who proved that the fermenting strain was not a contamination but was really derived from the original non-fermenting organism, and arose upon lactose-agar as a variant with a new character which bred true. Since then, numerous instances have been recorded by Burk,[11] Jacobsen,[12] Twort,[13] Müller,[14] and others, of members of the Colon-Typhoid group which displayed a similar capacity of varying towards one or more of the carbohydrates, and the fact of bacterial variation in this direction is now beyond dispute."

As the nutrient agar on which we grow our cultures is made from fresh beef-meat infusion containing the natural muscle sugars, and not from beef extract, it was thought that perhaps Bacillus abortivo-equinus quickly accommodated itself to an environment of lactose and acquired the ability to ferment this sugar. We have proved this not to be the case, not only by using cultures of the first generation, but by making inoculations of the lactose broth directly from the tissues of the original material. Dilutions of this same material in nutrient agar gave a pure culture of the organism. After the growth had fermented the lactose broth, dilutions from this broth in agar yielded, as far as we could determine, a pure culture, thus proving that the organism in some cases possesses as an original physiologic characteristic the ability to ferment lactose, and that the resultant gas-production is not caused by a contaminator.

[7] A Manual of Determinative Bacteriology, 1901, p. 207.
[8] A Text-book on Disease Producing Micro-organisms, etc., 1910, p. 452.
[9] A Text-book of General Bacteriology, 1914, p. 262.
[10] Centralbl. f. Bakteriol., I, O., 1913, 68, p. 151.
[11] Arch. f. Hyg., 1908, 65, p. 235.
[12] Centralbl. f. Bakteriol., I, O., 1910, 56, p. 206.
[13] Proc. Roy. Soc., 1907, 79, p. 329.
[14] Centralbl. f. Bakteriol., R., 1908, 42 p. 57.

The Smith fermentation tubes[15] were used in the first fermentations carried on by us. Since with these tubes it is possible to measure with precision the amount of gas formed in the upright arm, they are valuable in the quantitative determination of gas-production. The Smith tubes, however, require a large amount of media, and they are fragile, and cumbersome.

The inverted vial is a simple combination, merely a small inverted tube, sealed at one end, in a test tube. The handling of this device, as can readily be seen, is the same as that of a test tube, and it is therefore especially convenient for extended work with gas-production. It is not possible, however, to measure with much exactitude the amount of gas formed. However, in our experiments the vials were all made of the same caliber glass tubing and were all as near the same length as was possible with ordinary laboratory technic. The inverted tubes were therefore practically identical and exact comparisons could be made as to the amount of gas formed.

W. W. Brown[16] concludes from a comparative study of the Smith fermentation tube and the inverted vial in the determination of sugar-fermentation that inverted vials are more efficacious in the low dilutions than the fermentation tubes, and that fermentation tubes are more efficacious in higher dilutions than the inverted vials. Brown's conclusions are based on his work with oysters grown in beds polluted by city sewage. He inoculated his sugar media with varying amounts of shell liquor obtained from the oysters. In all of our work, the sugar media were heavily inoculated, and therefore, if Brown's conclusions hold true, with our technic the inverted vial was the more efficacious.

In order that we might satisfy ourselves as to the efficacy of the inverted vial in registering small amounts of gas, the two systems were given a thorough comparative test. Test tubes containing inverted vials and the regulation Smith fermentation tubes were filled with lactose broth of the same lot. The tubes, both the Smith tubes and the inverted vials, were inoculated with a strain of B. abortivo-equinus taken from the same subculture. A number of controls were also used; that is, several tubes of the media were not inoculated, but were incubated and otherwise treated in the same manner as those in which the organism had been placed. The tubes were incubated at 37.5 C. Observations made at the end of 24, 48, and 72 hours showed that

[15] Smith: The Fermentation Tube, 1893.
[16] Am. Jour. Pub. Health, 1913, 3, p. 701.

30% more of the inverted vials registered gas than of the Smith tubes, while all the control tubes of both kinds, remained negative.

By careful measurement we find that the average amount of gas produced in lactose broth by B. abortivo-equinus in Smith fermentation tubes, is about 2%. The amount of gas formed from lactose and saccharose sugars by B. abortivo-equinus was always very small. It was thought that possibly the small bubble arising after inoculation of the tubes with this organism was caused by a physiologic change in the medium, due either to the mechanical disturbance produced by whipping the broth with the platinum needle at the time of the introduction of the culture, or to expansion and later contraction of the contents of the inverted vial, caused by the difference in temperature between incubator and laboratory. The temperature of the incubator is always 37.5 C., while that of the laboratory varies between 20 and 25 C.

A long series of tests was made to determine whether the bubble was of physical or chemical origin. A number of tubes were inoculated with different strains of B. abortivo-equinus. A much larger number were whipped with a sterile platinum needle, the treatment being the same as that accorded the inoculated tubes, save the sterility of the inoculating needle. These tubes, together with a great many control tubes, were placed in the incubator. The control tubes were inverted vials filled with media identical with that in the inoculated tubes. The cotton plugs in these tubes were not removed from the time of sterilization of the tube and its contents until the test was over. The control tubes in this case were used as a check on the original media. The test was observed every day for several days, and usually most of the inoculated tubes showed the characteristic bubble, but in no instance was even a minute bubble observed in the tubes containing the media whipped with the sterile needle, or in the control tubes.

From this test we have concluded that the small bubble, which we are wont to term "the characteristic bubble," is without doubt the result of fermentation; altho very small, usually about 2% of the media being displaced, this bubble is the result of chemical change. Therefore we believe that B. abortivo-equinus does ferment lactose in a majority of trials, and saccharose, in some cases, to a slight degree, and that the characteristic bubble encountered in our tests is not of physical, but of chemical, origin.

Cultural media prepared by commercial companies are not used in our laboratory. The media for the tests are prepared by us according

to Jordan.[17] Five hundred grams of minced lean beef are placed in 1000 c.c. of distilled water and kept in the ice-box over night. The liquor is then strained—the juice having been well pressed out of the meat—and boiled for half an hour to coagulate the albumins. These are then filtered out. Meat usually contains a slight amount of muscle sugar. A simple method of removing the muscle sugar is that devised by Theobald Smith: From 10 to 20 c.c. of a pure young broth culture of B. coli are added to the infusion of meat and the whole incubated 18 hours at 37.5 C. The broth is then placed in the autoclave and subjected to from 20 to 25 pounds pressure to kill the organism. A series of fermentation tubes is filled with this supposedly sugar-free broth, inoculated with an active gas-producing B. coli, and incubated at 37.5 C. for 24 hours. If no gas is formed, we are reasonably certain that all the fermentable carbohydrates have been removed. The fluid is now made up to 1000 c.c. with distilled water, and 10 gm. of Witte's peptone are carefully stirred in and dissolved by heating. The broth, which at this stage is generally markedly acid, is then titrated and adjusted while hot to a neutral reaction by the addition of the required amount of a normal solution of sodium hydroxid. The broth is then heated in the autoclave to 120 C., allowed to cool again to bring down the precipitate caused by change of reaction, and filtered. The special broth media are now finally prepared by the addition of 1% of the desired sugar or other carbohydrate to the sugar-free broth. Five cubic centimeters of the special broth medium are pipetted into a tube containing an inverted vial and sterilized in the Arnold steam sterilizer by the discontinuous method for 3 successive days. The following sugars have been used in our fermentation tests: xylose, adonite, rhamnose, raffinose, arabinose, sorbose, sorbite, dulcite, dextrose, mannite, maltose, saccharose, and lactose. As the ability of B. abortivo-equinus to split the last two sugars named was questioned, the greater part of the work was done with saccharose and lactose.

All strains of B. abortivo-equinus used in these tests were isolated by this laboratory from cases of infectious abortion in mares in several different studs located in the Bluegrass Region. The strain of B. enteritidis (Gaertner) was secured from Dr. Biehn, of Chicago. The strain of the paracolon bacillus was presented to us by Dr. Surface, who personally brought the culture from the laboratory of Prof. Dr. C. O. Jensen, of Copenhagen. For convenience, the organisms will be

[17] A Text-book of General Bacteriology, 1912, p. 29.

referred to by number. Numbers 1 to 16 were Bacillus abortivo-equinus, isolated as follows:

1. From the aborted male fetus of a mare.
2. From the uterine exudate of an aborting mare.
3. From the aborted female fetus of a jennet.
4. From the aborted fetus of a mare.
5. From the fetal membranes of an aborting mare.
6. From the fetal membranes of an aborting mare.
7. From the aborted male fetus of a mare.
8. From the aborted male fetus of a mare inoculated experimentally with the organism.
9. From the aborted male fetus of a mare.
10. From the aborted male fetus of a mare.
11. From the uterine exudate of an aborting mare.
12. From the uterine exudate of an aborting mare.
13. From the aborted male fetus of a mare.
14. From the genital discharge of a mare giving birth to a very weak living foal, in a stud where infectious abortion existed.
15. From the uterine exudate of an aborting mare.
16. From the uterine exudate of an aborting mare.
17. A mixed culture of the Bacillus abortivo-equinus consisting of Strains 2, 3, 5, 6, 7, 11, 12, 13, and 15.

345. Bacillus enteritidis (Gaertner) secured from Dr. Biehn.

597. A paracolon bacillus isolated from a case of diarrhea in a calf. This bacillus had been secured from Dr. Surface.

By reason of careful laboratory practice we are comparatively certain that our observed results were caused by the inoculations and not by contamination. All glassware after being washed with soap and water is rinsed and dried and sterilized with dry air at 200 C. After being filled with the media the tubes are again sterilized by the discontinuous method in the Arnold steam sterilizer. Culture tubes are never opened, except in a dust-proof compartment especially constructed for this purpose, the walls of which are kept saturated with glycerin. In almost all instances the inoculations were made from 18-hour-old plain-agar cultures, the material being taken from the drop of condensation. Check tubes were used in every series of fermentation tests.

The following tables give the results of our fermentation tests. In Table 1 are presented observations made when 1% lactose broth was inoculated severally with the 16 different cultures of B. abortivo-equinus, and one (No. 17) mixed culture of Strains 2, 3, 5, 6, 7, 11, 12, 13, and 15. Table 2 gives observations made when 1% saccharose was used instead of 1% lactose. All observations were made after 60 hours' incubation at 37.5 C.

TABLE 1

GAS-PRODUCTION IN 1% LACTOSE BROTH BY DIFFERENT STRAINS OF BACILLUS ABORTIVO-EQUINUS

Strain	Total Number of Tests	Number of Tests Showing Gas	Number of Tests Showing No Gas	Average Percentage of Gas When Produced	Average Pecentage of Acid
1	14	8	6	1.5	.3
2	6	6	..	3.0	.3
3	12	6	6	1.5	.2
4	10	5	5	1.5	.2
5	6	6	..	3.0	.2
6	7	7	..	2.5	.4
7	5	5	..	3.0	.4
8	8	8	..	3.0	.5
9	5	5	..	3.0	.5
10	8	4	4	1.5	.2
11	4	4	..	2.0	.2
12	3	3	..	2.0	.3
13	5	3	2	2.0	.2
14	3	3	..	2.0	.2
15	4	4	..	2.0	.2
16	4	4	..	2.5	.2
17	12	12	..	5.0	.3
Total.....	116	93	23	...	...

TABLE 2

GAS-PRODUCTION IN 1% SACCHAROSE BROTH BY DIFFERENT STRAINS OF BACILLUS ABORTIVO-EQUINUS

Strain	Total Number of Tests	Number of Tests Showing Gas	Number of Tests Showing No Gas	Average Percentage of Gas When Produced	Average Pecentage of Acid
1	11	2	9	2.0	.2
2	4	2	2	1.8	.15
3	7	2	5	1.5	.15
4	4	1	3	2.0	.2
5	2	1	1	1.0	.2
6	2	1	1	1.2	.25
7	2	2	..	2.0	.2
8	3	1	2	1.5	.2
9	2	2	..	2.0	.25
10	5	2	3	1.8	.2
11	2	2	..	2.2	.25
12	2	1	1	2.5	.25
13	2	2	..	2.0	.2
14	2	1	1	1.5	.2
15	2	2	..	2.0	.2
16	2	2	..	2.0	.15
17	2	2	..	1.8	.2
Total.....	56	28	28	...	...

TABLE 3

COMPARISON OF BACILLUS ABORTIVO-EQUINUS, BACILLUS ENTERITIDIS (GAERTNER), AND THE PARACOLON BACILLUS IN REGARD TO GAS-PRODUCTION IN 1% LACTOSE BROTH

Organism	Source	Total Number of Tests	Number of Tests Showing Gas	Number of Tests Showing No Gas
B. abortivo-equinus.....	Aborting mares.....	116	93	23
B. enteritidis.....	Not known.....	9	6	3
Paracolon bacillus.....	Calf diarrhea.....	2	..	2

TABLE 4

COMPARISON OF BACILLUS ABORTIVO-EQUINUS, BACILLUS ENTERITIDIS (GAERTNER), AND THE PARACOLON BACILLUS IN REGARD TO GAS-PRODUCTION IN 1% SACCHAROSE BROTH

Organism	Source	Total Number of Tests	Number of Tests Showing Gas	Number of Tests Showing No Gas
B. abortivo-equinus.....	Aborting mares.....	56	28	28
B. enteritidis.....	Not known.....	7	1	6
Paracolon bacillus.....	Calf diarrhea.....	5	..	5

TABLE 5

GAS-PRODUCTION ON OTHER SUGARS

Sugar	B. Abortivo-equinus 1	B. Abortivo-equinus 3	B. Abortivo-equinus 4	B. Abortivo-equinus 10	B. Enteritidis (Gaertner) 345	Paracolon Bacillus 597
Xylose.....	+	+	+	+	+	+
Adonite.....	—	—	—	—	—	—
Rhamnose.....	—	—	—	—	—	—
Raffinose.....	+	+	+	+	+*	+*
Arabinose.....	+	+	+	+	+†	—
Sorbose.....	—	—	—	—	—	—
Sorbite.....	+	+	+	+	+	+
Dulcite.....	+	+	+	+	+	+
Glucose.....	+	+	+	+	+	+
Mannite.....	+	+	+	+	+	+
Maltose.....	+	+	+	+	..	..

The sign + indicates the production of gas; the sign —, no gas.
* Small amount of gas was noted after 3 days' incubation.
† An extremely small bubble of gas in one instance and no gas in another test.

From the tables it will be seen that of 116 tests with B. abortivo-equinus in lactose, 93 show an average of 2% gas-production and 23 no gas. With the same organism in saccharose, 28 of the tests are positive, showing a little less than 2% gas, while 28 are negative for gas but show a slight amount of acid, an average of about 0.3%.

The question now arises as to what the cause of this variance is. It would seem that if gas is produced in some instances, it would be in all cases, if the same organism is used and the same cultural medium

and technic are employed. This is not always the case, and if variants of this organism are not the cause, we are at the present time unable to suggest an explanation of the phenomenon. When our tests are made in duplicate and triplicate, the cultural medium is of the same lot, the inoculating material comes from the same culture, and the tubes are treated exactly alike; yet after incubation we often find that the duplicates differ, one being positive and the other negative. If any gas is produced, be it ever so slight, the test must be recognized as positive. When the test is made in triplicate, one of the tubes often fails to agree with the other two.

The culture of B. enteritidis (Gaertner) produced approximately 2% gas in lactose in 75% of the trials; in saccharose a slightly smaller amount of gas was produced in 1 of 7 trials. The strains of paracolon bacilli used did not produce gas in either lactose or saccharose.

In these tests the four strains of B. abortivo-equinus produced the following average percentages of gas in the carbohydrates which were fermented: xylose 51%; raffinose 39%; arabinose 59%; sorbite 82%; dulcite 95%; glucose 74%; mannite 81%. With these same materials, B. enteritidis (Gaertner) produced the following amounts of gas: xylose 30%; arabinose, a minute bubble in one instance and none in another; raffinose 8%; sorbite 85%; dulcite 5%; glucose 60%; and mannite 80%. The paracolon bacilli used produced 5% gas in xylose, 7% in raffinose, 85% in sorbite, 75% in dulcite, 60% in glucose, 80% in mannite and no gas in arabinose. In these tests no gas was produced by the four strains of B. abortivo-equinus used, or by B. enteritidis (Gaertner) and B. paracolon, in adonite, rhamnose, and sorbose.

From these tests, it would seem that arabinose, raffinose, and dulcite could be used to good advantage in differentiating B. abortivo-equinus from B. enteritidis (Gaertner) and the types of paracolon bacilli used, as in these tests only a most minute bubble of gas was formed in arabinose by B. enteritidis and none in another, and no gas was formed by the paracolon bacillus. An average of 40% gas was produced by B. abortivo-equinus in raffinose, while only 8% gas was produced in this medium by the other organisms. However, these results are not in accord with the general literature on the subject, in that raffinose is not fermented by organisms belonging to intermediate groups of the colon-typhoid group.[18] The fermentation in dulcite by B. abortivo-equinus is but little during the first 24 hours, after which it

[18] Besson: Practical Bacteriology, Microbiology and Serum Therapy, p. 434.

proceeds rapidly and in some cases to such an extent as to empty the medium in the inverted vial. Meyer[19] also states that the reaction on dulcite can be used for differentiation.

CONCLUSIONS

The inverted vial was as efficacious in our work as the Smith fermentation tube.

The bubble, or the small amount of gas, encountered so often in our fermentation tests with Bacillus abortivo-equinus in lactose and saccharose broth is not of physical, but of chemical, origin.

Bacillus abortivo-equinus produced approximately 2% gas in lactose in 80% of 116 trials, and in saccharose slightly less than 2% gas in 50% of 56 trials.

The average gas-production by the strain of Bacillus enteritidis (Gaertner) was about 2% in lactose in 80% of the trials, and a slightly smaller amount in saccharose in 1 of 7 trials. The strain of the paracolon bacillus used did not ferment lactose or saccharose, a fact which is in accord with the literature on the subject.

Bacillus abortivo-equinus may or may not produce gas in 1% lactose or saccharose broth, even varying in this respect in duplicate and triplicate tests.

Bacillus abortivo-equinus possesses as an original physiologic characteristic the ability, in most cases, to ferment lactose to a small extent, and also, in some cases, to ferment saccharose to a less extent. This characteristic in all probability has not as yet been accentuated by environment.

Lactose and saccharose broth can be employed to good advantage in laboratory routine for differentiating Bacillus abortivo-equinus from the colon bacillus, as the gas, when produced, is small in amount; and, in all probability, dulcite and perhaps raffinose can be used to advantage in differentiating Bacillus abortivo-equinus from other members of Subgroup II of the colon-typhoid group, but absolute proof as to its identity can only be secured through the use of other tests, such as those for further cultural characteristics and the complement-fixation and agglutination tests.

[19] Jour. Med. Research, 1913-14, 29, p. 325.